EXHIBIT 3

Vaccination of Sheep against *Fasciola hepatica* with Glutathione S-Transferase

Identification and Mapping of Antibody Epitopes on a Three-Dimensional Model of the Antigen[1]

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The glutathione S-transferases (FhGST) of the liver fluke *Fasciola hepatica* have been identified as novel vaccine candidates that protect sheep against a fluke infection. With the use of overlapping peptides covering the predicted amino acid sequences of four FhGST cDNAs, we have defined the linear epitopes recognized by polyclonal antibody from sheep vaccinated with FhGST. Dominant and minor epitopes were found to be present on all four of the sequences although some epitopes were shown to be specific to particular FhGST. A high percentage of the FhGST peptides were found to be antigenic although considerable variability in response to the peptides was observed among the animals. This analysis was extended to the IgG1 and IgG2 response at the peptide level. Based on the recently solved crystal structure of the rat mu-class GST 3–3, a three-dimensional model of one of the FhGST sequences was generated that allowed the predicted spatial localization of defined epitopes. Most epitopes were localized on regions of high flexibility and accessibility. A comparison of epitopes on FhGST with the B cell epitopes on Sm28, a 28-kDa GST from *Schistosoma mansoni*, has found few similarities. There was no correlation between an antibody response to linear peptide epitopes and the level of protection induced in sheep by vaccination with FhGST. *Journal of Immunology*, 1994, 152: 1861.

Glutathione S-transferases (GST)[4] are a family of multifunctional proteins that catalyze the conjugation of glutathione to electrophilic substrates. These GS-conjugates are thought to be substrates for the ATP-dependent GS-X export pump (1) or alternatively are the first step in the formation of mercapturic acids, classical water soluble excretion products (2). Four classes of mammalian cytosolic GST (alpha, mu, pi, and theta) have been described that show broad but overlapping substrate specificity (3). Recent advances in the area of crystal structure analyses of these enzymes has been significant. Since the first reported crystal structure of a pig pi-class GST in 1991 (4), the crystal structure of two of the classes, pi and mu, has been reported in different species (5, 6). This has allowed for the first time the ability to directly compare the structures from different classes and attempt to relate this to their overlapping but distinct substrate specificities[5].

The use of GST as an immunogen for vaccination of animals against parasitic diseases has been extensively characterized with a 28-kDa GST from *Schistosoma mansoni* (Sm28). Sm28 has been shown to consistently induce

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[4] Abbreviations used in this paper: GST, glutathione S-transferase; 3D, three-dimensional; FhGST, GST from *F. hepatica*; %HOD, percentage of the highest OD; Sm28, 28-kDa GST from *S. mansoni*.

[5] Wilce, M. C. J. and M. W. Parker. 1994. Structure and function of glutathione S transferases. *Biochim. Biophys. Acta.* (*In press*).

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a reduction in total parasite burdens against an infective challenge in many animal models including mice, rats, hamsters (7, 8), monkeys (9), and baboons (10). Synthetic peptides, selected on the basis of predictive algorithms, have been characterized for the presence of B and T cell epitopes (11). An octameric construct of one of the peptides mimicked the action of the whole protein by partially protecting rats after active immunization (12). In addition to the observed reduction in parasite numbers, immunization with Sm28 decreased both the fecundity and viability of eggs released by *S. mansoni* worms (10). The reduction in worm number and the effect on egg production are believed to be mediated by different effector mechanisms on the basis of a study using mAb to Sm28 (13). A recent study has localized the antifecundity effect to Ab raised against the carboxyl-terminus (peptide 190–211) and to a lesser extent the amino terminus (peptide 10–43) of Sm28 (14). Recently Trottein et al. (15) have reported the crystallization of Sm28. The solving of this structure will enable localization of both B and T cell epitopes on the three-dimensional (3D) structure as well as defining their proximity to the active sites on the enzyme.

An initial study using the GST from *Fasciola hepatica* (FhGST) as a vaccine candidate in rats failed to induce protection (16). In contrast, in our own studies we have significantly protected sheep by vaccination with the native FhGST (17). Four cDNA clones encoding FhGST have subsequently been isolated and sequenced and found to be homologous to the mu-class of GST (18). These recombinant proteins have been shown in vaccination trials to protect both sheep and cattle against fluke infection (Panaccia, M., S. Mailer, L. Salvatore, F. Bowen, T. Friedel, C. Morrison, and T. Spithill, unpublished observations). In our study, using a set of overlapping peptides predicted from the cDNA sequences of FhGST, we have been able to define the linear epitopes present on the four rGST from *F. hepatica* using Ab from sheep vaccinated with FhGST. With the aid of a 3D model of FhGST, based on the known crystal coordinates of the rat mu-class GST (5), we have localized these epitopes spatially on the predicted structure of the FhGST molecule.

This study has given an insight at the peptide level into the variability of the antibody response of individual vaccinated sheep to respond to a defined antigenic mixture. Importantly, we have analyzed the humoral immune response to native FhGST in vaccinated sheep in an attempt to identify peptide epitopes recognized by animals showing immunity to fluke infection.

Materials and Methods

Immune sera

A group of 10 Merino wethers, obtained from Henty, NSW, Australia, were immunized s.c. with 100 μg of native FhGST in CFA (CSL, Parkville, Australia) 8 wk before challenge. They received a boost of 100 μg of FhGST 4 wk before infection in IFA (CSL) and a third similar dose in PBS on the day before challenge. The sheep were infected intraruminally with 500 metacercariae, purchased from Compton Paddock Laboratories (Surrey, UK), suspended in a 0.4% w/v suspension of high viscosity carboxymethylcellulose (Sigma Chemical Co., St. Louis, MO).

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                1         10         20         30         40
GSTRAT:  PHILGYWNVRGLTHPIRLLLEYTDSSYEEKRYAMGDAPDYDRSQWLNEK
FhGST :  PAKLGYWKIRGLQQPVRLLLEYLGEEYEEHLYGRDDREK------WFGDK
Cons.     P  LGYW  RGL  P RLLLEY     YEE  Y   D        W   K
                                                  <.loop.>

                50         60         70         80         90
GSTRAT:  FKLGLDFPNLPYLIDGSRKITQSNAIMRYLARKHHLCGETEEERIRADIV
FhGST :  FNKGLDLPNLPYIIDGKCKLTQSVAIMRYIADKHGMLGTTPEERARIEMI
Cons.    F  GLD PNLPY ID   K TQS AIMRY A KH   G T EER R

               100        110        120        130        140
GSTRAT:  ENQVMDNRMQLIMLCYNPDFEKQKPEFLKTIPEKMKLYSEFLGKRPWFAG
FhGST :  EGAAMDLRMGFVRVCYNPKFEEVKGDYLKELPTTLKMYSNFLGDRHYLTG
Cons.    E   MD RM     CYNP FE  K   LK  P   K  S FLG R   G

               150        160        170        180        190
GSTRAT:  DKVTFVDFLAYDILDQYHIFEPKCLDAFPNLKDFLARFEGLKKISAYMKK
FhGST :  SPVSHVDFMVYEALDCIRYLAPQCLEDFPKLKEFKSRIEDLPKIKAYME
Cons.     V  VDF  Y  LD     P CL   FP LK F  R E K KI AYM S

               200        210
GSTRAT:  SRYLSTPIFSKLAQWSNK
FhGST :  EKFIKWPLNGWIASFGGGDAAPA
Cons.       P  S  A
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FIGURE 1. Sequence alignment of mu-class rat GST and *F. hepatica* GST51. Sequences predicted from cloned cDNAs for the rat (20) and *F. hepatica* (18) GST were aligned. Conserved residues (Cons.) have been defined through the sequence. The residue numbering is that of the FhGST51.

Sera were collected from these animals at 4-wk intervals and stored frozen at −20°C. Animals were slaughtered 16 wk postinfection and liver worm burdens were determined. One animal died during the course of the experiment. Native GST was purified as previously described (17).

Synthesis of peptides

Overlapping nonapeptides, initiating at every second residue, were synthesized from the amino acid sequences predicted from the four rGST cDNAs of *F. hepatica* (18) on cleavable-linker derivatized polyethylene pins (19). Biotin was coupled to an N-terminal extension (Gly-Ser-Gly-Ser-Gly) on each peptide. After side-chain deprotection and washing, the peptide was cleaved from each pin into 200 μl of 0.1 M sodium phosphate buffer, pH 7.2. Microtiter plates containing the cleaved peptide solutions were stored at −20°C until needed. The synthesis of the peptides was performed by Coselco Mimotopes (Clayton, Australia).

ELISA

Polyvinyl microtiter plates (A/S Nunc, Roskilde, Denmark) were coated overnight at 4°C with 5 μg/ml streptavidin (Sigma) in 0.1 M carbonate buffer, pH 9.3. Wells were washed with PBS, 0.05% Tween 20 between incubations. Unbound sites were blocked with PBS-Blotto (PBS, 0.05% v/v Tween 20, 5% w/v skim milk powder (Diploma, Bonlac Foods Ltd., Melbourne, Australia)) (200 μl/well) for 1 h. For this and all subsequent incubations, the plates were incubated at room temperature on a rocking platform and, unless otherwise stated, a volume of 100 μl was added to each well. Biotinylated peptides were diluted 1/500 in PBS, 0.05% v/v Tween 20, 0.1% w/v BSA, and incubated for 1 h on the plates. The plates were incubated for another 1 h with a 1/1000 dilution of sheep sera. For this step and all subsequent dilutions of Ab, PBS-Blotto was used as diluent. Total bound sheep Ig was measured with the addition of a 1/500 dilution of affinity purified donkey anti-sheep Ig conjugated to horseradish peroxidase (Silenus, Melbourne, Australia) for 1.5 h. Alternatively, specific IgG1 or IgG2 Ab subclasses were measured with the addition of mAb to sheep IgG1 and IgG2. These mAb were generously supplied by Dr. Ken Beh (CSIRO, McMaster Laboratory, Sydney, Australia). Bound mAb was detected with the addition of an affinity purified rabbit anti-mouse Ig conjugated to horseradish peroxidase (Silenus) for 1.5 h. Color development was achieved with the addition of 100 μl of 0.001 M 2,2-azinobis (3-ethylbenzthiazole sulfonic acid) in 0.062 M citric acid/0.076 M Na_2HPO_4 pH 4.0, 0.03% v/v hydrogen peroxide. The OD was measured at 414 nm with an automated Titertek Multiskan spectrophotometer (Flow Laboratories Australasia, Nth Ryde, Australia).

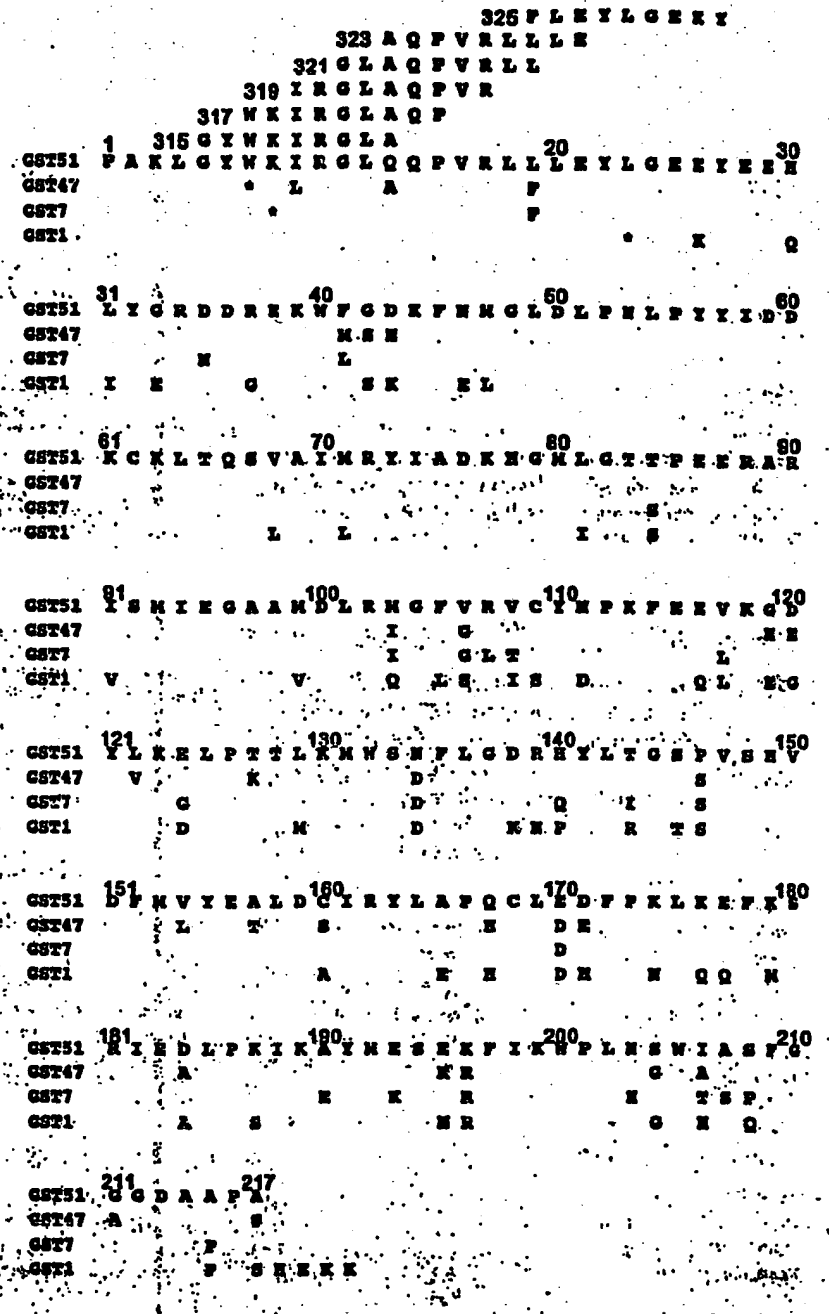


FIGURE 2. Amino acid sequence of four rGST from *F. hepatica* showing the sequence of rGST51 in full and sequence differences in rGST47, rGST7, and rGST1. An asterisk denotes the beginning of the protein sequence predicted from the rGST47, rGST7, and rGST1 cDNAs. A set of overlapping nonapeptides, initiating at every second residue, was synthesized from the sequence of rGST51 and regions that differ in rGST47, rGST7, and rGST1. An extra six peptides (peptides 315–325) were synthesized to cover possible N-terminal polymorphisms in the unknown N-terminus of rGST1 from *F. hepatica*.

ODs corresponding to bound Ab are expressed as a percent OD calculated as follows. The overall nonspecific background contributed by the individual serum was determined by the OD in wells containing no peptide. This background OD was subtracted from the ODs obtained in the wells containing peptide to give a corrected OD. Each corrected OD was then expressed as a percentage of the highest OD for a given serum obtained against any of the peptides tested in the ELISA and termed %HOD.

Construction of F. hepatica GST model

FhGST (rGST51 (18)) has an amino acid identity of 45% with the rat mu-class GST (20). A comparison of the tertiary structures of homologous proteins have found that 3D structures show a higher degree of conservation in evolution than primary structure (21). The 3D structure of the rat mu-class GST has recently been determined (5) and thus the rat mu-class GST structure was used to build the core framework of the FhGST51 model. The rat mu class GST coordinates were extracted from the Brookhaven Protein Database (Brookhaven National Laboratory, Upton, NY) and the sequences of the *Fasciola* and rat mu-class GST were aligned by hand as shown in Figure 1. The program HOMOLOGY (Version 2.0 (1992), BIOSYM Technologies, Inc., San Diego, CA) was used to assign the coordinates of the rat mu-class GST to the FhGST51 in the following manner. Where the side chains of the rat mu-class and FhGST51 were the same, the complete set of coordinates, including both the backbone and sidechain, were transferred to the FhGST51 model. Where the rat mu-class and FhGST51 side chains differed, only the peptide backbone coordinates were transferred. Coordinates for nonconserved side chains were taken from an amino acid rotamer library and positioned in the model in the same orientation as the replaced amino acid residue. There is a five residue deletion in the FhGST51 sequence, beginning at residue number 40, relative to the rat GST sequence (Fig. 1). To permit flexibility in this region, residues 38 to 40 of the FhGST51 model were not assigned coordinates from the rat mu-class GST. The coordinates of these three residues were generated by manually building

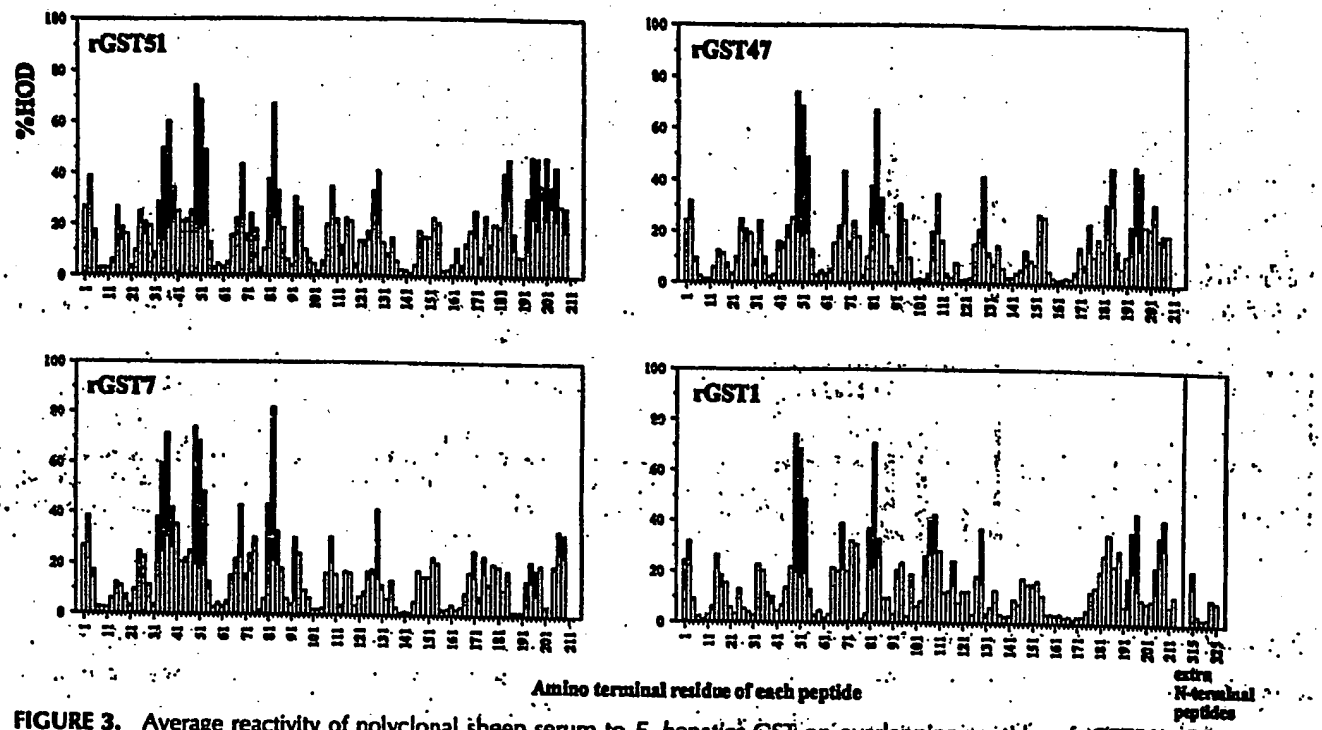


FIGURE 3. Average reactivity of polyclonal sheep serum to *F. hepatica* GST on overlapping peptides of rGST51, rGST47, rGST7, and rGST1. The height of each black bar represents the average %HOD of nine animals for each peptide. One SD (*open bar*) has been included to indicate the variability between sera in response to that peptide.

a loop between residues 37 and 41. The C-terminal extension of the FhGST51 were assigned coordinates from an amino acid rotamer library and put into an extended conformation.

This initial model was modified to reduce close contacts between side chain atoms. This was achieved by manually adjusting the dihedral angles. A complete energy minimization was performed upon the model using the program DISCOVER (Version 2.8 (1992), BIOSYM), with 500 cycles of the steepest descent method and 1500 cycles with the conjugate gradient method. The surface accessibility and the secondary structure of the FhGST51 model were calculated using the program DSSP (22).

Results

Analysis of linear epitopes on GST of F. hepatica

Overlapping nonapeptides (initiated at every second amino acid) were synthesized to represent all the possible linear epitopes predicted from the cDNA sequences of four GST from adult *F. hepatica* (18) (Fig. 2). These proteins have been termed rGST51, rGST47, rGST7, and rGST1. All peptides covering the predicted amino acid sequence of rGST51 were synthesized whereas only those peptides with sequence differences to rGST51 were synthesized from the predicted primary structures of rGST47, rGST7, and rGST1. Although the rGST51 protein was predicted from a complete cDNA, the other FhGST cDNAs were incomplete and did not encode the N-termini (Fig. 2) (18). The N-terminal sequence was determined by amino acid sequencing of the entire pool of affinity isolated FhGSTs (23). These data revealed highly conserved N-termini but three sites of polymorphism were observed at positions 9, 13, and 19. The rGST7 and rGST47 sequences cover this region being truncated by only seven and eight amino acids, respectively. However, the rGST1 sequence begins 22 residues short of the demonstrated N-terminus. In our study, we have assigned the amino acids Leu, Gln, and Leu for positions 9, 13, and 19, respectively, in rGST1. These nominations were deduced from the high frequency of rGST1 transcripts in an adult cDNA library (Panaccio, M., unpublished observations) and the relative high ratios of Leu, Gln, and Leu, respectively, at the polymorphic amino acid positions observed by N-terminal sequencing of native GST (23). In the event that these assumptions were incorrect, an additional six peptides (315–325) were synthesized to cover the remaining potential sequences arising from the polymorphic sites. The peptides start from the Pro, not the Met (residue 1 in Panaccio et al. (18)), because the N-terminal sequence analysis of native GST from adult parasites did not identify the Met at residue 1 (23).

Polyclonal sera, collected 4 wk after challenge with *F. hepatica*, from nine sheep immunized with native GST were assayed for their reactivity to the panel of peptides. This group of vaccinated sheep was part of a larger vaccine trial investigating the vaccination potential of native GST from adult *F. hepatica* under differing vaccination regimes. The highest level of protection in the trial was seen in this group with a 44% reduction ($p < 0.001$) in worm burden relative to the infected control animals (Sexton, J., M. Panaccio, S. Mailer, L. Salvatore, G. Wijffels,

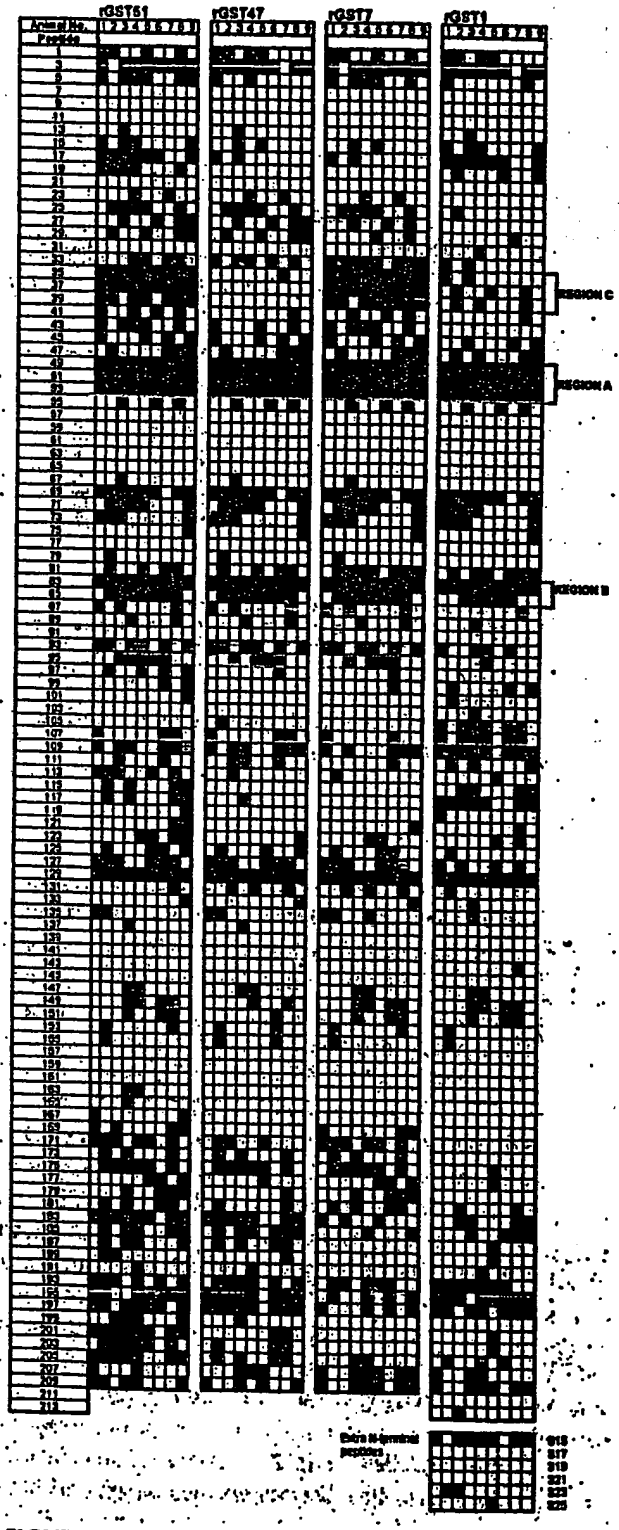


FIGURE 4. Total Ab reactivity of polyclonal sheep serum to *F. hepatica* GST from nine individual animals on the overlapping peptides of rGST51, rGST47, rGST7, and rGST1. High reactivity (%HOD >40) is denoted by a *black square*, with a *gray square* representing reactivity above background (15 < %HOD < 40). A *white square* indicates reactivity below background (%HOD < 15). The animals are in order of increasing worm burden from left to right with worm burdens of 40, 122, 124, 172, 235, 242, 270, 317, and 406, respectively. The mean worm burden in the control sheep in this trial was 378. Thus, animals 1, 2, 3, and 4 showed greater than a 50% reduction in worm burden relative to the controls.

L. Wilson, N. Cambell, C. Thompson, and T. Spithill, unpublished observations). The decision to analyze sera from wk 4 postchallenge was influenced by the biochemical findings in our first trial that suggested that killing or retardation of the challenge parasites had occurred early in their migration from the intestine and through the liver parenchyma (17). The anti-GST Ab response in these sera (titers of 1/200,000–1/1,000,000) originates predominantly from vaccination because it has been shown by ELISA analysis that infection with *F. hepatica* induces only a very low anti-GST Ab response (<1/100) under our experimental conditions (data not shown). This is in agreement with the results of Hillyer et al. (24). We have found that to obtain a peptide scan with sufficient sensitivity to detect the minor epitopes identified below, antibody titers of more than 1/10,000 are necessary.

The average reactivity of the sera to each of the FhGST peptides is shown in Figure 3. The height of the black bar represents the mean %HOD for each peptide obtained by averaging the response from the nine animals in the trial. One SD (height of the open bar) has been included to indicate the variability in reactivity between sera. Therefore, a black bar indicates a consistently recognized response to that peptide. In contrast, high variability in the response to a peptide is indicated in Figure 3 solely by a white bar that demonstrates the SD is equal to or greater than the mean. Comparison of the peptide scans for each of the GST proteins indicates that reactivity is present along the length of the four rGST sequences. The regions of highest antigenicity are concentrated in the N-terminal half and the C-terminus. The response to many of these peptides is highly variable as demonstrated by the large SD. Often the SD was equivalent to the average response to that peptide. A similar analysis was undertaken with sera from our first trial (17) with comparable results. Due to the lower GST Ab titers in sera from this trial (data not shown), it was not possible to complete our study with these sera.

To determine if a particular linear epitope correlated with a reduction in worm burden, the reactivities of individual sera were further analyzed. For clarity, the responses to each of the rGST has been presented in a different format (Fig. 4) to allow a direct comparison of the reactivity of the nine animals to this peptide set. High reactivity was set at above 40%HOD (*black square*) and lower reactivity established as above 15%HOD and below 40%HOD (represented by a *gray square*). Fifteen percent was chosen as the cut-off for a positive reaction as comparison of repeat assays on individual sera found that the greatest variation in reproducibility was observed below this level. The data in Figure 4 are presented with the animals

Table I. *Shared and specific epitopes identified on rGST*

Epitope Class[a]	Type[b]	Epitope	rGST51	rGST47	rGST7	rGST1
Shared	Dominant (region A)	49–61	+++	+++	+++	+++
	Dominant (region B)	83–93	+++	+++	+++	+++
	Minor	3–11	+	+	+	+
	Minor	69–77	++	++	++	++
	Minor	127–137	+	+	+	+
Specific	Dominant (region C)	35–47	+++	−	+++	±
	Minor	17–25	+	±	±	+
	Minor	105–117	±	±	±	+
	Minor	117–125	±	−	−	+
	Minor	183–193	+	+	±	±
	Minor	195–205	++	++	±	++
	Minor	201–213	++	±	±	±

[a] Each epitope has been classified as present on each of the four GST (shared epitope) or on a particular rGST (specific epitope).
[b] The average reactivity of the nine antisera to each epitope has been defined as a dominant response (>50 %HAb) or a minor response (15 < %HAb > 50).
[c] The level of reactivity of each of the epitopes on each of the four rGST has been scored to reflect the differences between the epitopes. The scoring system indicates +++ as high, ++ as medium, + as low, − as below background and ± to indicate low but variable responses between the animals to that epitope.

(nos. 1–9) ranked in order of increasing worm burden from left to right. The data show that there is no peptide(s) to which an antibody response correlates with a reduction in worm burden. We have extended this analysis with sera from a total of 89 sheep from different native GST vaccine trials and have not found a total Ab response to a linear epitope that correlates with protection (data not shown).

Definition of shared epitopes on rGSTs of F. hepatica

An epitope has been defined on the basis of reactivity by all or most of the sera using the data from Figure 4. A dominant epitope has an average reactivity of more than 50%HOD whereas a minor epitope is more than 15%HOD and less than 50%HOD. Five antigenic regions are found on all four rGSTs, not necessarily as a consequence of identical sequences (Figs. 3 and 4); these have been defined as shared epitopes. The two most dominant epitopes with the highest level of reactivity are present first on amino acids 49–61, which we have termed region A and second on amino acids 83–93, termed region B. The amino acid sequence of region A is identical in all four rGST although the sequence of region B differs among the four rGST (Fig. 2). Three other minor epitopes at amino acids 3–11, 69–77, and 127–137 are consistently recognized by the majority of animals on each rGST but with a lower level of reactivity. A summary of epitopes shared among the different rGST is presented in Table I.

Definition of epitopes specific to particular rGST of F. hepatica

We have defined specific epitopes as those epitopes that are found on particular rGSTs (Fig. 4). The most dominant of these epitopes, termed region C, is present on amino acids 35–47 in rGST51 and rGST7. The difference in reactivity between the four rGST at this epitope appears to be due to the amino acid changes at residues 41, 42, and 43 in which a Phe/Leu(41), Gly(42), or Asp(43) in rGST51 and rGST7 correlates with reactivity although little or no reactivity was observed in rGST47 and rGST1 with a Met/Phe, Ser, and Glu/Lys at positions 41, 42, and 43, respectively. Minor specific epitopes are present on amino acids 17–25 on rGST51 and rGST1; 105–117 and 117–125 on rGST1; 183–193 on rGST51 and rGST47; 195–205 on rGST51, rGST47, and rGST1; and 201–213 on rGST51. The region 169 to the C-terminus of all the rGSTs is highly antigenic but the pattern of reactivity is not identical among the rGSTs. It is interesting to note that the C-terminal four amino acid extension on GST1 (211–221) is not antigenic. Epitopes specific to particular rGST are summarized in Table I.

The above epitopes have been defined on the basis of reactivity by all or most of the sera. However, many other peptides are antigenic to a smaller number of sera. Overall 80% of the peptides of rGST51 are antigenic (i.e., recognized by one or more sera). Similarly, 68% of the peptides in rGST47 and rGST1 and 66% in rGST7 are antigenic. The average analysis of all the peptides on the four rGST shows that 71% of the peptides are antigenic.

3D model of rGST51 of F. hepatica

A 3D model of FhGST51 was built based on the recently solved crystal structure of the mu-class rat GST (5). A Ramachandran plot (25) of the backbone dihedral angles for the FhGST51 model illustrated that all the residues are in the frequently observed angle ranges for proteins (data not shown). The epitopes defined in Table I were mapped onto the model with the following color coding: red-dominant shared epitope; orange-minor shared epitope; dark blue-dominant specific epitope; and yellow-minor specific epitope. The amino acid backbone is displayed in turquoise. Figure 5 displays the epitope localization on the monomer (Fig. 5*b*) and dimer (Fig. 5*c*) representation of the FhGST51 model with the residue numbering indicated

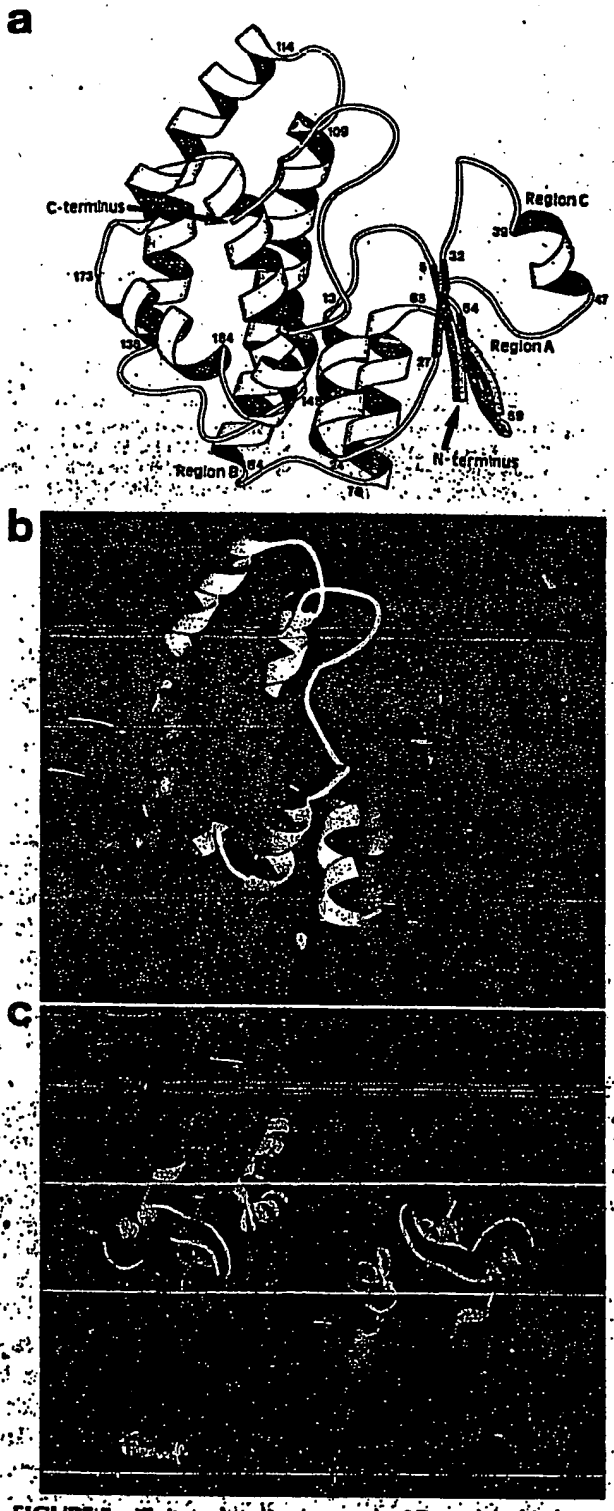


FIGURE 5. Epitope localization on the 3D model of *F. hepatica* rGST51. A schematic representation of the monomer is shown in (*a*) to define the orientation of the molecule. Epitopes defined in Table I were localized on the monomer (*b*) and dimer (*c*) configuration of the 3D model of FhGST51 with the following color coding: *red*-dominant shared epitope, regions A and B; *orange*-minor shared epitope; *dark blue*-dominant specific epitope, region C; and *yellow*-minor specific epitope. *Turquoise blue* represents the backbone of the molecule.

on the schematic (Fig. 5*a*) representation of the model. Each of the epitopes defined in Table I localize to both loops and secondary structural features such as α-helices and β-strands except that two of the minor shared epitopes at amino acids 69–77 and 127–137 that lie only on α-helices. Regions A and B from different monomers were shown to lie in close proximity (<8 Å apart) in the dimeric model (Fig. 5*c*).

Comparison of epitopes with amino acid surface accessibility and temperature factors

Epitopes have most frequently been observed at regions of proteins that are surface accessible (26) and thus most methods for predicting the location of epitopes are based on this observation. The surface accessibility of the FhGST51 model is shown in Figure 6. Another commonly used parameter to define epitopes is localized flexibility (27–29). The crystallographically determined temperature factor can be used as an indicator of main chain flexibility. The main chain temperature factors of the rat mu-class GST aligned with the FhGST51 sequence are shown in Figure 6. Wilce and Parker[5] have shown that the temperature factors of GSTs from three different mammalian classes (alpha, mu, and pi) are similar. This, coupled with the high sequence homology of the *Fasciola* and rat mu-class GST, indicate that the rat mu-class temperature factors provide a reasonable estimate to approximate the flexibility of the main chain of the FhGST51 molecule.

Two of the major epitopes, regions B and C, are found at positions of the FhGST51 model with high surface accessibility and flexibility. The other major epitope, region A, is in an area of the model that is not exposed to the solvent and is not predicted to have high main chain flexibility. All of the minor epitopes, except on amino acids 69–77, are found at positions of high surface accessibility and flexibility.

Characterization of IgG1 and IgG2 isotypic antibody responses on overlapping peptides of GST of F. hepatica

The isotypic specificity of the antibodies cross-reacting with each of the peptides in eight of the sera was determined using mAb specific for sheep IgG1 and IgG2 (Fig. 7). In previous studies, vaccination with native GST in sheep has been shown to induce both a IgG1 and IgG2 subclass Ab response (data not shown). Total IgA and IgM Ab levels were found to be extremely low in sera (data not shown) and it was not possible to determine their specificity at the peptide level.

To determine subclass specificity at each of the epitopes, triplicate assays for total Ab as well as IgG1- and IgG2-specific Ab were performed concurrently. It was therefore possible to directly assign reactivity for each of the peptides on a scan for total Ab to a contribution by IgG1 and/or IgG2 Ab. As seen in Figure 7 only a small number of peaks (shown in *green*) could not be correlated

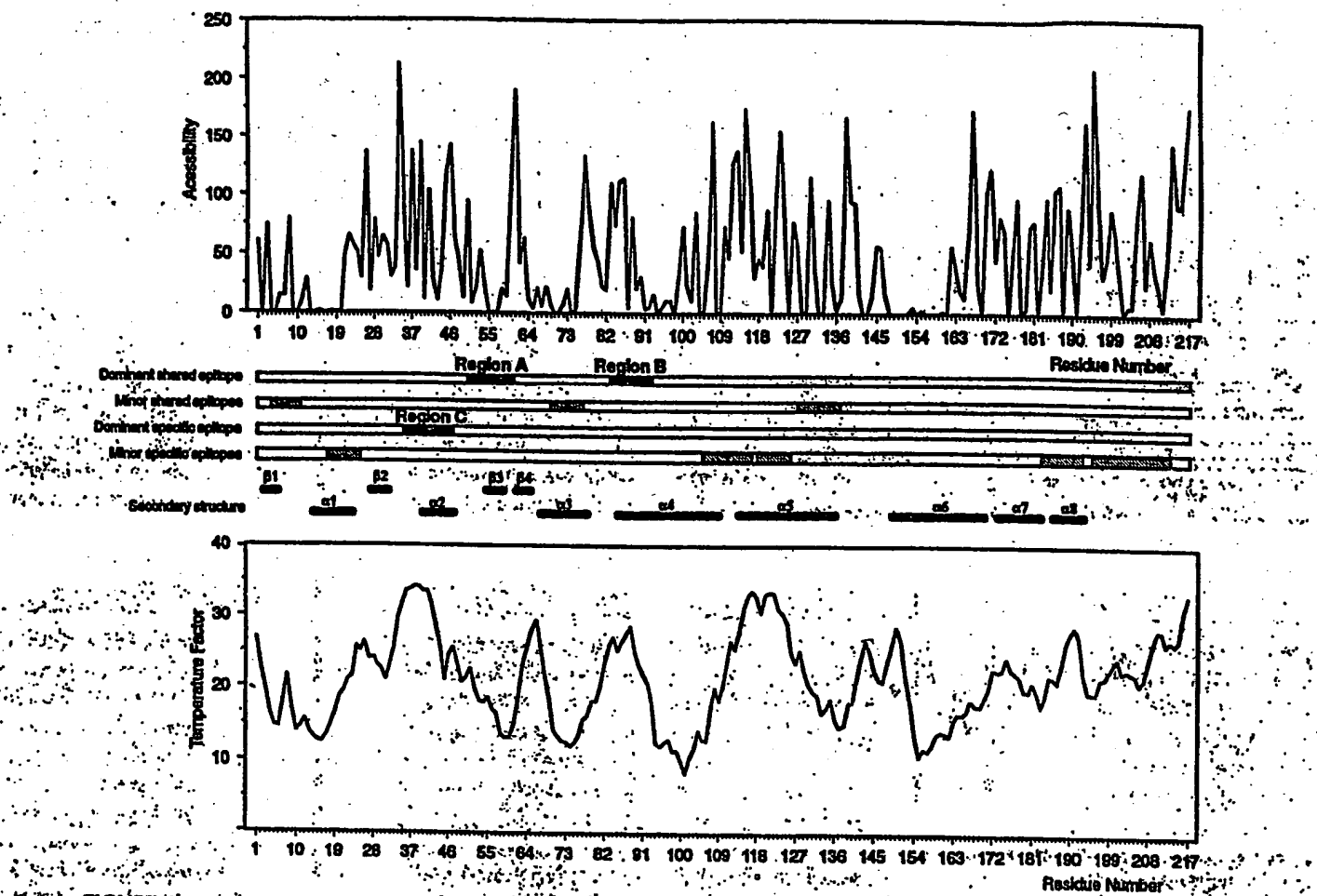


FIGURE 6. Comparison of surface accessibility of residues in the 3D model of *F. hepatica* rGST51 and temperature factors of the mu-class rat GST with the predicted epitopes of *F. hepatica* rGSTs. The predictive capacity of surface accessibility and flexibility to define regions of antigenicity are compared with epitopes defined by Ab reactivity. The secondary structure of the FhGST51 model, defining regions of α-helices and β-strands, is based upon the nomenclature defined in Reinemer et al. (4).

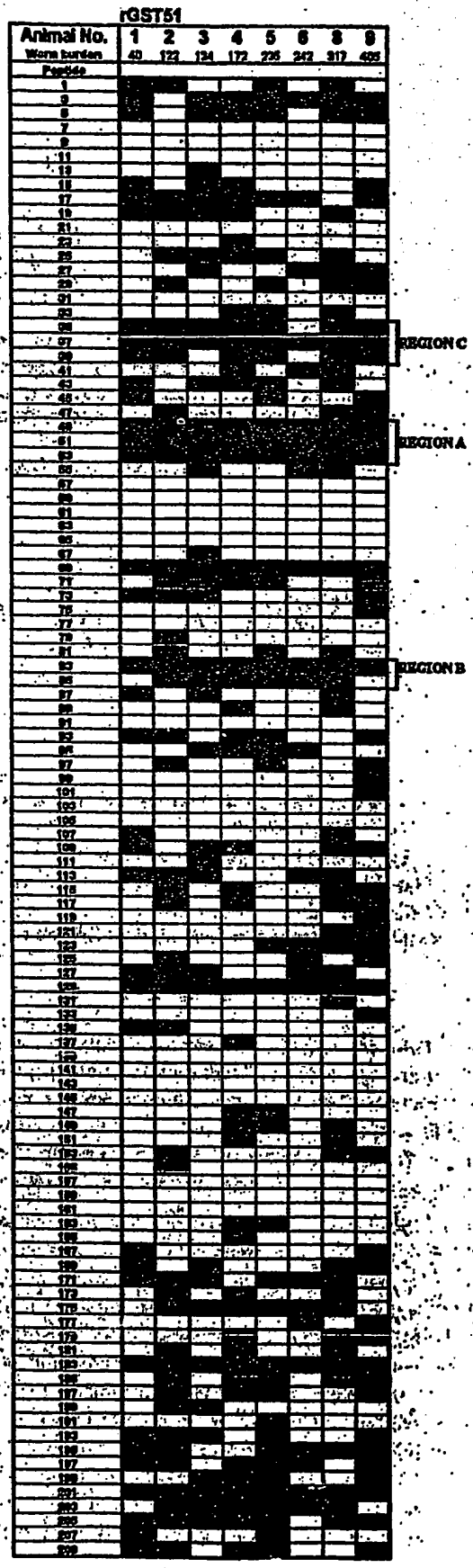
with an IgG1 and/or IgG2 response. The dominant epitopes at regions A, B, and C were antigenic to both IgG1 and IgG2 Ab (*black*). Generally, most peptides were recognized by Ab of the IgG1 isotype (*purple*) with only a limited number of IgG2 subclass-specific responses (*blue*) with the exception of animal 3 that was dominated by IgG2-specific epitopes.

A limited number of responses (shown in green in Fig. 7) could not be accounted for by IgG1 or IgG2 subclass Ab. This suggests the involvement of Ab of a different subclass, possibly IgE, IgG3, or IgG4 although there is no evidence for the presence of IgG3 and IgG4 subclass Ab in sheep. IgE has been implicated in immunity to helminth infection (30), exemplified by the correlation of an IgE response with protective immunity in rats and humans infected with *S. mansoni* (31). The cDNA sequence of sheep IgE has been cloned and specific reagents for the detection f sheep IgE are under development (32).

There was no correlation between the subclass-specific response at the peptide level with the differences in worm burden from each of the animals.

Comparison of defined epitopes on Sm28 GST of S. mansoni with GST of F. hepatica

The peptide epitopes of Sm28 recognized by Ab from vaccinated mice and rats and infected humans (33–37), were compared with the homologous regions in FhGST51 (Table II). Major B cell epitopes of Sm28 have been extensively characterized on peptides 24–43, 115–131, and 140–150. The homologous region of the Sm28 peptide 24–43 on FhGST contained the N-terminus of the dominant epitope at region C on rGST51 and rGST7 and the C-terminus of a minor epitope (17–25) on rGST51 and rGST1. For the region on FhGST homologous to the Sm28 peptide 115–131, reactivity was present only on rGST1 at the C- and N-termini, respectively, of the minor specific



epitopes at 105–117 and 117–125. Little or no reactivity was seen in the homologous region of FhGST for peptide 140–150 in our sequences (residues 133–144 in FhGST). Antibodies of the IgA subclass were present in human infection sera to the peptide 158–175 and the C-terminal peptide, 190–211, of Sm28 (35). The low IgA Ab titer induced in response to vaccination of sheep with FhGST precluded determining if the C-terminus of FhGST similarly induced an IgA response. However, the C-terminal region of all four rGST are highly antigenic to both IgG1 and IgG2 Ab (Fig. 7 and data not shown).

Discussion

Although research on the enzyme family of GSTs has been extensive, only recently has the 3D crystal structure of these proteins been elucidated (4–6). In the absence of these data, the very extensive study on the antigenicity and immunogenicity of Sm28 from *S.mansoni* has defined likely regions of B and T cell epitopes on the basis of predictive algorithms (11). In our study, we have been able to define the linear B cell epitopes present on four rGST from *F. hepatica* with the use of overlapping nonapeptides cross-reactive with sheep antisera raised to native GST and subsequently localize these epitopes on a 3D model of a GST from *F. hepatica*.

The antigenicity of the four rGSTs is high in sheep, with 80% of the peptides of rGST51 (68% rGST47, 66% rGST7, and 68% rGST1) reacting with at least one or more of the nine antisera, correlating well with an accessibility profile that predicts that many of the residues are surface located. This is consistent with the hypothesis that the entire surface of a molecule is intrinsically antigenic and the sites that elicit an immune response are a function of the bias of the immune response of the immunized host (38, 39). The variability in the pattern of reactivity seen between animals highlights the need to compare the responses of a large number of animals to determine a true analysis of the antigenicity of a protein in any particular host. The averaged reactivity of nine animals shown in Figure 3 is consistent with an averaged profile of a more extensive analysis of sera from 89 animals to native FhGST (data not shown). The analysis presented for only nine animals is thus representative of a much larger sample pool.

Comparable studies defining the antigenic regions of other protein Ag using overlapping peptides, finds varying levels of reactivity. In a study on *Neisseria* gonococcal C30 strain pilin (40), rabbit antiserum to the native protein cross-reacted with peptides along the entire length of the

FIGURE 7. Ab isotype reactivity of polyclonal sheep serum to *F. hepatica* GST from eight individual animals on the overlapping peptides of rGST51. Reactivity of different Ab isotypes with a peptide has been defined as both IgG1 and IgG2 (*black*), IgG1 only (*purple*), IgG2 only (*blue*), or unknown isotype (*green*).

Table II. *Comparison of defined B cell epitopes on peptides of 28-kD GST from S. mansoni with homologous regions on GSTs from F. hepatica*

			S. mansoni		*F. hepatica*
			B cell epitope		B cell epitope (defined in Table I)
Sm28 Peptides	Homologous FhGST Peptide	% Identity	Human sera[a]	Rat and mouse immune sera[b]	Sheep immune sera[c]
24–43	20–39	30	IgE, IgG4, IgA	IgG (mouse)	Minor specific epitope (17–25) on rGST51 and rGST1
				IgG2a (rat)	Dominant specific epitope, Region C, (35–47) on rGST51 and rGST7
115–131	111–124	18	IgE, IgG4, IgA	IgG (mouse)	Minor specific epitope (105–117) on rGST1
				IgG2a, IgG2c (rat)	Minor specific epitope (117–125) on rGST1
140–150	133–144	14	IgE, IgG4, IgA, IgG3	IgG, IgE	Poorly recognized
158–175	149–166	17	IgA	ND	Poorly recognized
190–211	189–210	14	IgA	ND	Minor specific epitope (195–205) on rGST51, rGST47 and rGST1
					Minor specific epitope (201–213) on rGST51

[a] Sera from a human population infected with *S. mansoni* (33, 34).
[b] Sera from rats and mice vaccinated with Sm28 (32, 35, 36).
[c] Sera from sheep vaccinated with native FhGST.

molecule. In the same study, 30% of peptides, encoding the sequence N-terminal to the trypsin cleavage site of the polyvirus coat protein JGMV-JG, cross-reacted with rabbit antisera raised to the whole virus, a total of nine antigenic regions in 90 amino acids. Contrastingly, seven regions of reactivity representing only 17% of peptides encoding dihydrofolate reductase cross-reacted with a pool of ($n = 2$) rabbit antiserum to this protein (41). Similarly, sera from cattle infected with *Mycobacterium bovis* had exceedingly low reactivity to the overlapping peptides of MPB70, a major secreted *M. bovis* protein, despite high Ab titers to the native protein (42). One major region was recognized by three infection sera, but only one serum displayed reactivity, albeit low, to other regions along the protein length.

Localization of epitopes on the 3D model has shown that the dominant epitopes lie on both loops and secondary structural features such as α-helices and β-strands. Many of the secondary structural features are less surface accessible and it is likely that the loop regions are antigenically more important. Antigenic sites on α-helical regions tend to be located on short stretches of helix or at the periphery of helices, corresponding to regions expected to have a higher degree of flexibility (28). Despite the finding of major epitopes on loops (regions A, B, and C), areas classically defined with high accessibility and flexibility, not all the loop structures are strongly antigenic (peptides 136–148 and 171–173) (Fig. 5). This highlights the failure of defining epitopes solely on the basis of these parameters.

The region of the 3D model corresponding to region A was found to be of consistently high antigenicity but was in a region of the 3D model that exhibited both low flexibility and surface accessibility. This could be due to errors in the model, although this would be unlikely due to the high sequence identity of the rat mu-class and FhGST51. This region has also been shown to have low flexibility and surface accessibility in the alpha- and pi-class structures, implying the same structural characteristics are observed in all classes (see footnote 5). It is most likely that this epitope occurs because of conformational variations of the FhGST molecule. From examination of the temperature factors of a range of GST structures, it has been shown that helix 2 (region C, marked dark blue in Fig. 5) is extremely flexible (see footnote 5). If this helix were to move, it would expose region A of the FhGST molecule and this would account for its high antigenicity. Further evidence that suggests this helix could move has been provided in a study on a human π-class GST (43). A disulfide bond was described between a cysteine in helix 2 (domain 1) with another cysteine in domain II that is more than 18Å away in the crystal structure (4), thus highlighting the flexibility of this region.

Nearly all of the antigenic regions detected by an anti-sheep Ig reagent could be accounted for by an IgG1 and/or IgG2 isotype Ab response. This is consistent with our previous findings that immunization with native GST in CFA in sheep induces both an IgG1 and IgG2 isotype response (data not shown). The dominant epitopes at regions A, B, and C generally cross-react with both isotypes. The limited number of epitopes with reactivity only to IgG2 Ab in Figure 7 was believed to be influenced by the lower titer of IgG2 and/or the lower sensitivity of the assay. An analysis of the average reactivity of IgG2 Ab found a comparable profile as seen for total Ab in Figure 3 except with a much lower level of reactivity (data not shown). The observation of both IgG1 and IgG2 Ab reacting with similar peptides is consistent with the finding of multiple Ab isotypes in

human sera cross-reacting with the same peptide from Sm28. However, with these data and our study, it was not possible to determine if the same critical residues were cross-reactive for each Ab isotype.

Potential antigenic regions on Sm28 were predicted from hydrophilicity, accessibility, and mobility plots. Peptides that cover a large proportion of the total sequence were extensively characterized for the presence of both B and T cell epitopes in both animal models and against human infection sera (11). A comparison of the sequences of the Sm28 peptides with the homologous regions of *F. hepatica* finds few similarities in reactivity. This may be a direct consequence of the limited sequence homology between the two species (<30% in the peptides shown in Table II). Sm28 is a chimera of both alpha and mu homologous sequences (44) whereas the GSTs from *F. hepatica* share greatest homology with the mu-class of mammalian GST (18). It may also highlight differences in reactivity as a consequence of the 3D location of a peptide including accessibility of those peptide sequences in the globular protein. Most striking was the absence of equivalent B cell epitopes in FhGST as observed on Sm28 on peptide 140–150. The equivalent region in FhGST (133–144) has high surface accessibility but is poorly antigenic. These differences may be due to the variation between host species in the repertoire of B cell responses (sheep in our study vs mice, rats, and humans).

Analysis of subclass Ab reactivity for the Sm28 GST and the individual Sm28 peptides has found a relationship between susceptibility and resistance to infection to *S. mansoni* in humans (34). Studies in the rat have identified IgE and IgG2a responses to peptides 24–43 and 115–131, as responsible for observed Ab-dependent cellular cytotoxicity to the larvae of *Schistosoma* in the presence of eosinophils as effector cells and IgG2c is known to block this effect (33). Ovine IgE has recently been cloned (32) and monospecific reagents for its immunochemical detection are eagerly awaited. A comparison of the sheep IgE response to peptides of FhGST will reveal whether such a response correlates with the level of protection observed in sheep.

In our study, it was not possible to correlate an antibody response to a peptide(s) with protection, at both the total antibody and the IgG1 and IgG2 isotype level. This is despite many of the shared and specific epitopes, including regions A and C, containing residues believed to lie within the glutathione and hydrophobic binding sites as defined by x-ray crystallography of the pig lung and human placenta π-class GSTs and the rat mu-class GST (4–6). For example, Trp38, Lys42, Leu50, and Pro51 are known to be associated with binding of glutathione at the active site of the pig, human and rat GSTs. These residues are conserved in *F. hepatica* and are located in the dominant epitopes at regions A and C. Similarly, Tyr7, shown to be essential in catalysis (45–47), is conserved in *F. hepatica* and is located within a minor epitope on all four of the FhGST. The definition of epitopes within or near the active sites suggests that binding f antibody could inhibit the enzyme activity of the molecule. However, antibody binding to active site residues does not correlate with protection and in recent in vitro studies we were unable to inhibit the enzymatic conjugation of 1-chlor-2,4-benzene to glutathione by native FhGST using these polyclonal sera (Chris Morrison, personal communication). The latter finding may reflect the high affinity FhGST has for these substrates and current studies are aimed at investigating the ability of the antisera to inhibit a range of electrophilic substrates with lower affinity for the enzyme. Despite the failure to demonstrate inhibition with these antisera, studies with mAb to Sm28 have shown that enzyme inhibition is not essential for inducing a reduction in worm burden against *S. mansoni* (13). It will be interesting in future studies to determine whether antibody-mediated inhibition of enzyme activity is important for the observed reduction in worm burden in vaccinated sheep and, moreover, to determine the role of Ab in the immunity of sheep to *F. hepatica*.

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